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16. ABSTRACT Three forms of Union Internationale Contre le Cancer (UICC) asbestos, amosite, crocidolite, and chrysotile, were assayed for their cytotoxicity (inhibition of colony formation) in cell culture. Using embryonic human intestine-derived (I-407) and adult rat liver-derived (ARL-6) epithelial cells, the order of cytotoxicity was chrysotile > amosite > crocidolite. All three asbestos types were more toxic to I-407 than to ARL-6 cells. Chrysotile, amosite, and crocidolite were also tested for inhibition of colony formation in cultures of mouse colon-derived epithelial-like (MCE-1) cells; these cells were more resistant than the I-407 cells to all three fiber types; although similar to the ARL-6 cells in sensitivity to chrysotile and amosite, the MCE-1 cells were more sensitive to crocidolite than the ARL-6 cells. Leaching of the three asbestos forms for 3 days in sterile deionized water did not appreciably affect the cytotoxicity of I-407 or ARL-6 cells. Leaching in hydrochloric acid, however, greatly decreased the cytotoxicity of chrysotile on these cells and particularly in cultures of ARL-6 cells slightly increased the cytotoxicity of amosite and crocidolite. Leaching in deionized water altered the composition of the asbestos as revealed by release of substantial levels of Mg^{2+} and Ca^{2+} into the leaching fluid. Greater titers of these ions were released during leaching in hydrochloric acid.

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Comparative Toxicities of Different Forms of Asbestos in a Cell Culture Assay[1]

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Three forms of Union Internationale Contre le Cancer (UICC) asbestos, amosite, crocidolite, and chrysotile, were assayed for their cytotoxicity (inhibition of colony formation) in cell culture. Using embryonic human intestine-derived (I-407) and adult rat liver-derived (ARL-6) epithelial cells, the order of cytotoxicity was chrysotile > amosite > crocidolite. All three asbestos types were more toxic to I-407 than to ARL-6 cells. Chrysotile, amosite, and crocidolite were also tested for inhibition of colony formation in cultures of mouse colon-derived epithelial-like (MCE-1) cells; these cells were more resistant than the I-407 cells to all three fiber types; although similar to the ARL-6 cells in sensitivity to chrysotile and amosite, the MCE-1 cells were more sensitive to crocidolite then the ARL-6 cells. Leaching of the three asbestos forms for 3 days in sterile deionized water did not appreciably affect the cytotoxicity of I-407 or ARL-6 cells. Leaching in hydrochloric acid, however, greatly decreased the cytotoxicity of chrysotile on these cells and particularly in cultures of ARL-6 cells slightly increased the cytotoxicity of amosite and crocidolite. Leaching in deionized water altered the composition of the asbestos as revealed by release of substantial levels of Mg^{2+} and Ca^{2+} into the leaching fluid. Greater titers of these ions were released during leaching in hydrochloric acid.

INTRODUCTION

Asbestos is of grave concern as a health hazard because of its cytotoxic, fibrogenic, and carcinogenic properties (Selikoff, 1974, 1977; Harington, 1976). Inspired asbestos fibers are found in the respiratory system, and in addition, these fibers can migrate across internal membranes and have been detected in thoracic and abdominal lymph nodes, liver, spleen, pancreas, and kidney (Godivin and Jagatic, 1970; Langer *et al.*, 1974). Fibers also infiltrate the gastrointestinal tract via pulmonary clearance (Evans *et al.*, 1973) and as a result of ingestion of asbestos-contaminated water and certain commercially prepared beverages, foods, and drugs (Cunningham and Pontefract, 1971, 1973; Pontefract and Cunningham, 1973; Nicholson, 1974; Speil, 1974). Data indicate that asbestos penetrates the gastrointestinal mucosa (Westlake *et al.*, 1965; Pontefract and Cunningham, 1973; Wagner *et al.*, 1973; Volkheimer, 1974; Westlake, 1974; Storeygard and Brown, 1977) and produces biochemical changes in the mucosa and lumen of the rat small intestine (Jacobs *et al.*, 1977, 1978). Exposure to asbestos is associated with an increased incidence of bronchogenic carcinomata and pleural and peritoneal mesotheliomata in humans (Bogovski *et al.*, 1973; Selikoff, 1977). An

[1] This work was supported by U.S. Environmental Protection Agency Grant R803998-01; parts of this study are available in Research Rep. EPA-600/1-79-023 (Reiss *et al.*, 1979).

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increased incidence of cancers of the gastrointestinal tract in asbestos workers has also been reported (Selikoff, 1974).

The biological effects of asbestos have been studied in several *in vivo* models (Morgan *et al.*, 1971; Stanton and Wrench, 1972; Wagner *et al.*, 1973, 1974; Kanazawa *et al.*, 1979a, b; Kawai, 1979). The experiments of Stanton and Wrench (1972) and Stanton *et al.* (1977) which demonstrated a similar although lesser fibrogenic and carcinogenic potential for glass fibers than for asbestos fibers suggested that the biological effects of asbestos might be related to structural rather than physicochemical properties.

In vitro studies, which facilitate a more detailed investigation of the toxic and carcinogenic mechanisms of asbestos fibers revealed that chrysotile was more toxic than the amphiboles, amosite and crocidolite, in cultures of macrophages (Allison, 1971; Bey and Harington, 1971; Miller and Harington, 1972; Wade *et al.*, 1976). Hemolysis as well occurred more readily in the presence of chrysotile than the amphiboles (Schnitzer and Pundsack, 1970; Desai *et al.*, 1975; Harington *et al.*, 1975). Asbestos also inhibited mass growth of epithelial cells in culture (Neugut *et al.*, 1978; Frank *et al.*, 1979; Wade *et al.*, 1979) and produced a decreased epithelial cell cloning efficiency (Brown *et al.*, 1978; Chamberlain and Brown, 1978). Although the mass growth studies indicated chrysotile to be more toxic than the amphiboles, the results of the cloning efficiency assays showed a reversal of this order of toxicity.

In order to study the cytotoxic effects of chrysotile and the amphiboles on cells of the alimentary tract and to clarify the conflicting data from earlier studies on the relative toxicities of these fibers, we examined the biological effects of the Union Internationale Contre le Cancer (UICC) standard reference samples of chrysotile, amosite, and crocidolite in cultures of human embryonic intestine-derived epithelial (I-407) cells and mouse descending colon mucosal epithelial-like (MCE-1) cells. Rat liver-derived epithelial (ARL-6) cells, a well-characterized cell line with a substantial capability for metabolism of xenobiotics (Williams, 1976; Tong and Williams, 1980) were also studied. Greater vulnerability of this cell line would indicate an activation requirement for asbestos; alternately, greater resistance would indicate the susceptibility of a toxic component of asbestos to metabolic inactivation. The assay described in this report differs from assays previously used and was found to be an improved method for the accurate evaluation of asbestos toxicity.

Using this cytotoxicity assay we report that the order of asbestos toxicity in all the cell lines tested was chrysotile > amosite > crocidolite. The cell lines, however, differed in their sensitivity to asbestos. Leaching for 3 days in sterile deionized water did not appreciably affect the cytotoxicity of the asbestos; however, leaching in hydrochloric acid decreased the cytotoxicity of chrysotile and slightly increased the cytotoxicity of amosite and crocidolite. During leaching, Mg^{2+} and Ca^{2+} were released from the asbestos fibers into the leaching fluid.

MATERIALS AND METHODS

Asbestos Samples

The UICC standard reference samples provided by the EPA were Canadian chrysotile B, crocidolite, and amosite (Timbrell and Rendall, 1971–1972). These

samples contain heterogeneous distributions of fibers up to 200 μm in length. Several parameters have been determined for these samples by Timbrell (1969): for example, the total (internal and external) surface areas estimated by nitrogen absorption are 5.7 ± 0.3, 8.3 ± 0.5, and 26.8 ± 0.7 m^2/g for amosite, crocidolite, and chrysotile, respectively. The external surface areas derived from permeability data, however, are 3.3, 3.1, and 4.9, respectively. The macroscopic effects of heat, hot dilute (50:50) HCl, and formamide on the fibers were also studied. Following short-term (10 min) exposure to dilute HCl, amosite and crocidolite were unchanged; however, chrysotile, which is more soluble in HCl and less stable than the amphiboles (IARC, 1973, 1977), exhibited a small decrease in refractive index from approximately 1.559 to 1.520.

Samples of asbestos were exposed to 130 $erg/mm^2/sec$ ultraviolet light for 2 hr before weighing in order to suppress viable contamination in a manner that would be least likely to alter the physical or chemical properties of the asbestos fibers. In order to eliminate inaccuracies in weighing caused by the electrostatic character of asbestos, aliquots of asbestos were weighed in a known weight of water or culture medium immediately prior to use.

Cell Cultures

All cells were maintained at 37°C in Williams' Medium E (Williams and Gunn, 1974) (WME, Flow Laboratories, Rockville, Md.) supplemented with 10% fetal bovine serum (Flow Laboratories) and containing 50.0 units/ml mycostatin (Gibco, Grand Island, N.Y.) and 100 μg/ml gentamycin (Schering, Kenilworth, N.J.).

Human intestine-derived I-407 (Henle and Deinhardt, 1957) and adult rat liver-derived ARL-6 (Williams, 1976) cells were used for most of the studies in this project. MCE-1 cells, derived from mouse colon, were isolated and maintained in this laboratory as described below. These cells were also used for cytotoxicity assays, but, as yet, have not been fully characterized.

For the isolation of the MCE-1 cells, the descending colon of 6- to 12-week-old neomycin- and mycostatin-pretreated mice was excised and slit longitudinally in order to expose the mucosal surface. Three- to four-centimeter sections of the colon were placed, with the serosal surface down, on Millipore filters of small pore size (0.2 μm) in 60-ml plastic tissue culture dishes, and 5 ml of Medium E containing 0.25% pronase were added. Following incubation with the enzyme for 25 min at 37°C, the pronase was removed, and the mucosal cells were detached by gentle scraping into fresh medium. A flexible rubber policeman was used to detach cells loosened by enzyme digestion and then a rigid wooden spatula was used to remove more tightly bound cells. Isolated cells were pelleted at 50*g* for 4 min and washed twice. Yields of 1.5×10^6 viable cells (85–95% trypan blue negative)/cm colon were obtained.

Primary cultures from the colon mucosa were established by seeding the cells into 35-mm plastic tissue culture dishes at a concentration of 6×10^5 cells/ml medium. Although the usual volume of medium added to these plates was 2 ml, during the establishment of primary cultures, a volume of 3 ml was used to improve the attachment efficiency of the mucosal cells. To further improve attachment, WME was supplemented with 20% fetal bovine serum. Soybean trypsin inhibitor (40 μg/ml, Sigma Chemicals, St. Louis, Mo.), a protease inhibitor, also was

added. The effectiveness of this inhibitor in promoting mucosal cell attachment may be attributed to inactivation of residual pronase activity. Using this methodology, an attachment efficiency of 10–20% was obtained; the cell population that attached was a mixture of epithelial- and fibroblast-like cells. In order to enrich the cultures with epithelial cells, beginning one week after seeding, 50 units/ml collagenase were added to the culture every 2 weeks for a period of 24 hr. This relatively low concentration of collagenase digested collagen fibers produced by invading fibroblasts and thereby facilitated epithelial cell growth. Once primary cultures were at least 80% confluent, they were trypsinized and transferred. One of the clones developed from these cultures was the MCE-1 cell line.

Cytotoxicity Assays

The cytotoxicity of asbestos samples was quantified by measuring the inhibition of I-407, ARL-6, and MCE-1 epithelial cell colony formation following exposure to asbestos. For this assay, 10 cells/cm² I-407 or MCE-1 cells or 20 cells/cm² ARL-6 cells were inoculated into 25 cm² culture flasks. Twenty-four hours after the cells were seeded, the medium was replaced with medium containing asbestos. Following 3 days of exposure to asbestos, the cells were washed twice and reincubated in asbestos-free medium. Between 1 and 2 weeks after the initiation of treatment, the cells were fixed in 10% formalin and stained with Giemsa for the determination of colony formation.

For experiments in which ARL-6 and I-407 cells were subjected to two intervals of exposure to asbestos, cells were incubated with asbestos for a 6-day interval and then washed twice. Cells exposed to the highest concentration of asbestos were reseeded; after 24 hr, these cells were reexposed to asbestos for a second 6-day interval. Colony formation following the first and second intervals of asbestos exposure were compared.

Leaching of UICC Asbestos

Approximately 50-mg samples of UICC asbestos (chrysotile, amosite, or crocidolite) were weighed in a premeasured aliquot of sterile deionized water or 5 N HCl. Each weighed sample was further diluted to 0.5% (w/v), stirred thoroughly to disperse the fibers throughout the fluid phase, and leached for one 96-hr or three consecutive 24-hr intervals at 25°C.

Following leaching, the asbestos samples were pelleted by centrifugation at 10,000*g* for 15 min, and the supernatants (except approximately 0.5 ml directly above the pellet) were removed. The asbestos pellets were resuspended in the remaining fluid and adjusted to the proper concentrations for quantification of asbestos toxicity. When three consecutive leachings were performed, each leaching was followed by centrifugation and resuspension of the asbestos pellet (in sterile water or 5 N HCl after the first two leachings and in culture medium after the final leaching). Before suspension in culture medium, the 5 N HCl samples were neutralized with 1 N NaOH. As a control, unleached asbestos samples were weighed in a preweighed aliquot of culture medium and adjusted to concentrations comparable to the leached asbestos. Following this protocol, no significant loss of asbestos weight occurred during leaching. This was established in control experi-

ments in which weighed samples of asbestos were leached for one 96-hr or three consecutive 24-hr intervals, dried at 50–75°C, and reweighed in a known weight of water.

Mg^{2+} and Ca^{2+} Determinations

Atomic absorption spectrophotometry was utilized for the determination of Mg^{2+} and Ca^{2+} concentrations in the postleaching supernatants. Both unfiltered aliquots and aliquots of supernatant filtered through a Nalgene 0.2 or 0.45 μm filter unit were analyzed.

RESULTS

Cytotoxicity of UICC Asbestos

The colony-forming efficiencies of untreated ARL-6 and I-407 cells were 50 and 40%, respectively. Exposure of these cultures to chrysotile, amosite, or crocidolite after attachment resulted in an inhibition of colony formation that was dependent upon dose and duration of treatment (Figs. 1 and 2). The order of toxicity was chrysotile > amosite > crocidolite. Although periods of exposure to the asbestos fibers were extended to include 7 days, cumulative data indicated that exposure for 3 days induced reliable and consistent toxic inhibition. For example, after treatment for 3 days with 5.0×10^{-4}% chrysotile, the colony-forming efficiency was reduced to 10 ± 5% of that in control cultures in four repeated experiments. In the ARL-6 line (Fig. 1) at least fivefold greater concentrations were required to produce inhibition of colony formation comparable to that in the I-407 line (Fig. 2).

The colony-forming efficiency of untreated mouse colon-derived MCE-1 cells was 60 to 75%. When exposed to chrysotile, amosite, or crocidolite and then assayed for colony forming inhibition, the MCE-1 cells were less sensitive to all fiber types than the I-407 cells (Fig. 3). Although the MCE-1 cells exhibited a level of susceptibility to chrysotile and amosite similar to that of the ARL-6 cells, they were more sensitive to crocidolite than was the ARL-6 line. Thus, for chrysotile, the order of cell line susceptibility was I-407 > ARL-6 ≥ MCE-1; for amosite, I-407 > MCE-1 ≥ ARL-6; and for crocidolite, I-407 > MCE-1 > ARL-6. In comparison to both the ARL-6 and MCE-1 cells, the I-407 cells were particularly susceptible to amosite and crocidolite.

To examine the mechanism of inhibition of colony formation by asbestos, studies were performed to determine whether the results of our cytotoxicity assay were affected by an alteration in the surface of the culture flasks produced by interaction with the asbestos fibers. In these experiments, flasks without cells were exposed to an equivalent concentration (10^{-3}%) or equivalent cytotoxic activities of chrysotile (2.5×10^{-4}%), amosite (2.5×10^{-3}%), and crocidolite (2.5×10^{-3}%) in WME and incubated for 3 days at 37°C. Following incubation, these flasks were washed twice to remove the fibers and seeded with I-407 cells at colony-forming density. The CFE of cells grown in chrysotile-pretreated flasks compared to that of cells grown in untreated flasks was unchanged (Table 1), but the CFE of cells grown in amosite- or crocidolite-pretreated flasks was decreased. Microscopic examination revealed that two washes had removed essentially all of the chrysotile fibers from the flasks, but the culture surfaces of flasks pretreated

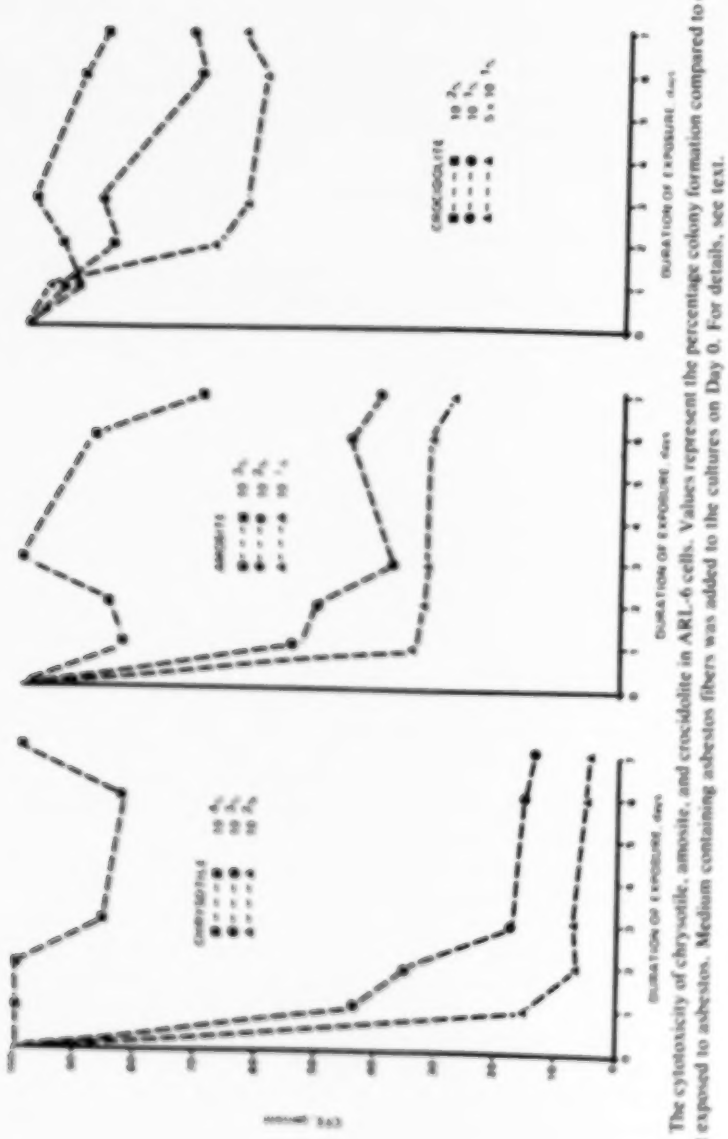

FIG. 1. The cytotoxicity of chrysotile, amosite, and crocidolite in ARL-6 cells. Values represent the percentage colony formation compared to control flasks not exposed to asbestos. Medium containing asbestos fibers was added to the cultures on Day 0. For details, see text.

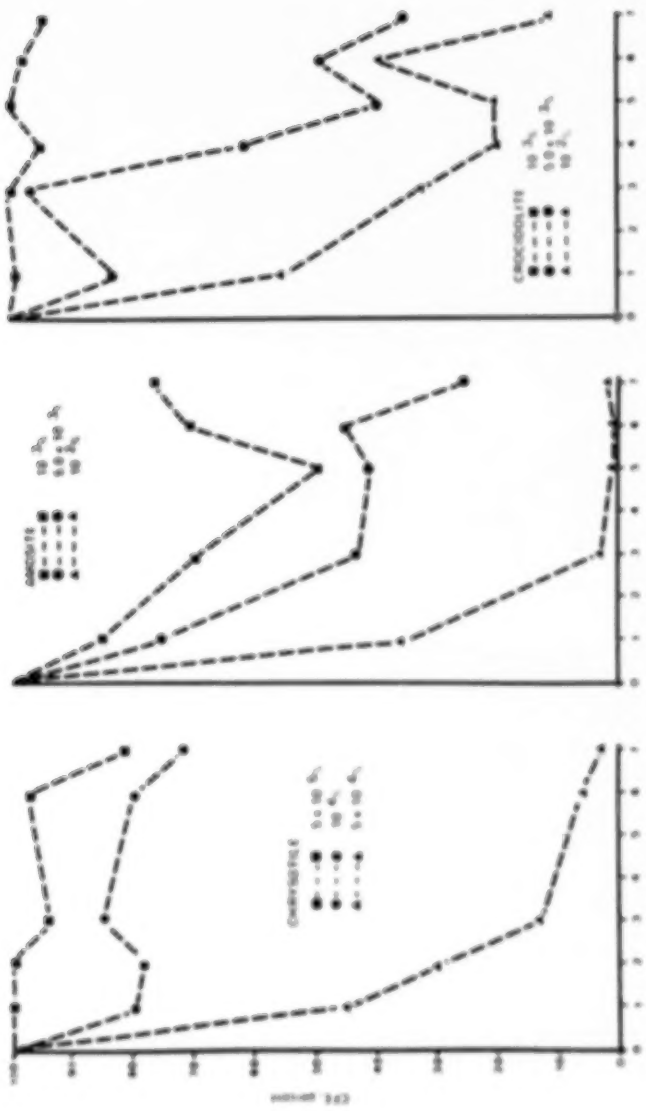

FIG. 2. The cytotoxicity of chrysotile, amosite, and crocidolite on L-407 cells. For details, see Fig. 1 and text.

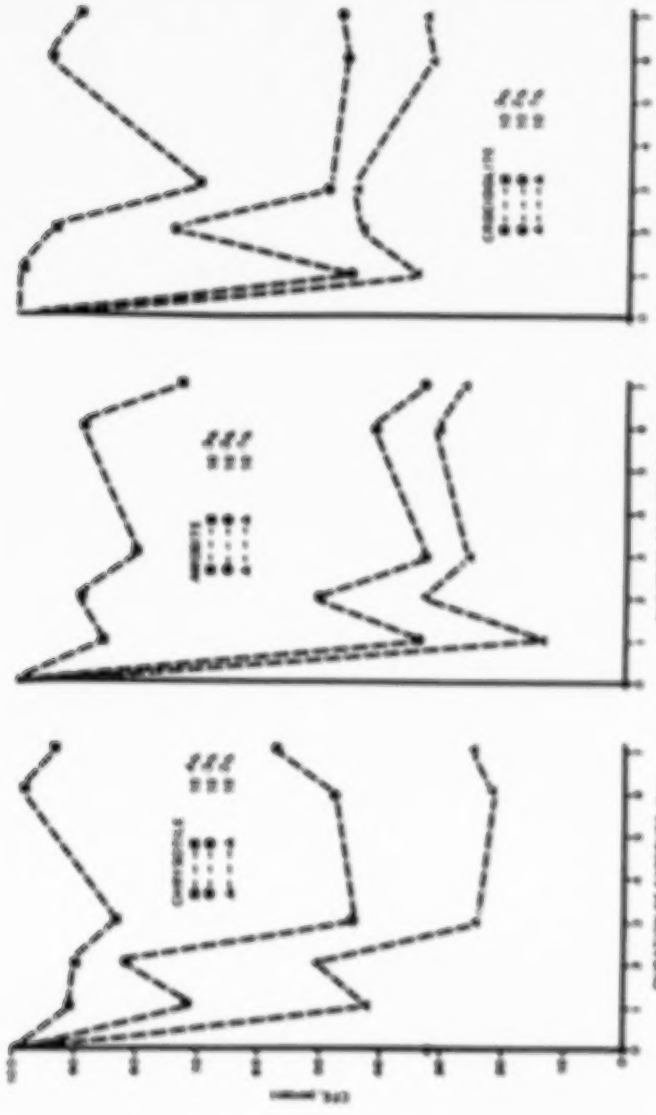

FIG. 3. The cytotoxicity of chrysoidin, ammonio, and crocodeline on MCF-1 cells. For details, see Fig. 1 and text.

TABLE 1

THE EFFECT OF ASBESTOS PRETREATMENT OF THE CULTURE SURFACE ON COLONY FORMATION OF I-407 CELLS[a]

Asbestos pretreatment	Relative colony forming efficiency (%)[b]		
	Washed twice	Washed twice with vigorous shaking	Washed three times and scraped twice
Chrysotile			
2.5×10^{-4}%	91 ± 8	—	100 ± 4
10^{-3}%	—	—	97 ± 7
Amosite			
10^{-4}%	—	—	100 ± 12
2.5×10^{-4}%	66 ± 4	75 ± 11	91 ± 7
Crocidolite			
10^{-4}%	—	—	99 ± 13
2.5×10^{-4}%	80 ± 5	100 ± 9	98 ± 7

[a] Medium E containing chrysotile, amosite, or crocidolite was incubated in 25-cm² flasks for 3 days at 37°C. Following incubation, the medium and fibers were removed by washing, washing and shaking, or washing and scraping with a rubber policeman. I-407 cells (10 cells/cm²) were then seeded onto the culture surface, and colonies were permitted to develop for 2 weeks before counting.

[b] Each value represents the average percentage colony formation for six flasks compared to six control flasks not exposed to asbestos. The standard deviations are included.

[c] Significantly greater than value for flasks washed twice with $P < 0.05$.

with the amphiboles, particularly amosite, still retained visible fibers. Both washing and vigorous shaking were required to remove the crocidolite fibers; in order to remove the more tightly adhered amosite fibers, alternate washing and scraping of the culture surface with a rubber policeman were necessary. Following the removal of essentially all of the fibers, the CFE of cells grown in asbestos-pretreated flasks was not changed compared to the CFE of cells grown in untreated flasks, indicating that asbestos did not affect the ability of the culture surface to sustain cell growth.

These results, however, suggested that adherence of asbestos to the culture surface could limit growth. To examine this possibility, asbestos was added, together with or 24 hr after the I-407 cells, and the differential effect on colony formation was determined. Amosite fibers produced a greater inhibition of colony formation when added at the time of seeding than when added 24 hr after seeding (Table 2). Chrysotile, however, exerted the same toxicity under both conditions. Thus, the presence of the highly adherent amosite fibers during the 24-hr attachment interval following seeding seemed to inhibit cell attachment, thereby reducing the number of colonies formed and increasing the apparent toxicity. In contrast, the less adherent chrysotile fibers did not interfere with cell attachment.

In order to verify that inhibition of colony formation by asbestos fibers added subsequent to cell attachment represents an accurate measure of asbestos toxicity and not a result of growth limitation due to the presence of adherent fibers on the culture surface, I-407 and ARL-6 cells surviving a 6-day interval of exposure to equivalent activities of chrysotile, amosite, and crocidolite were washed twice

TABLE 2
I-407 Colony-Forming Efficiency following 2 Days of Exposure to Amosite or Chrysotile Initiated during Seeding or 24-hr after Seeding[a]

Concentration (%)	Relative colony-forming efficiency (%)[b]	
	Fibers added during seeding	Fibers added 24 hr after seeding
Amosite		
5.0×10^{-1}	63 ± 4[c]	78 ± 4
10^{-2}	36 ± 13[c]	73 ± 9
2.5×10^{-2}	28 ± 1[c]	37 ± 4
5.0×10^{-3}	1 ± 1[c]	8 ± 2
Chrysotile		
2.5×10^{-4}	34 ± 6	35 ± 1
5.0×10^{-4}	13 ± 4	11 ± 3
10^{-3}	5 ± 1	4 ± 2

[a] I-407 cells (10 cells/cm^2) were seeded into 25-cm^2 flasks together with or 24 hr before the addition of amosite or chrysotile. Following exposure, the cultures were washed twice, and colonies were permitted to develop for 2 weeks before counting.

[b] Each value represents the average percentage colony formation for three flasks compared to three control flasks not exposed to asbestos; the standard deviations are included. Confirmatory results were obtained in another similar experiment.

[c] Significantly less than value for fiber addition at 24 hr with $P < 0.05$.

and, following the development of countable colonies, were trypsinized and reseeded at colony-forming density. This procedure effectively diluted asbestos fibers remaining after the second wash 1000-fold. Despite the absence of fibers during the second seeding, the number of colonies of chrysotile-exposed cells was substantially reduced; the number of colonies of amphibole-exposed cells was reduced, but to a lesser extent. Inhibition of colony formation by chrysotile in these experiments must be attributed to the toxicity of prior chrysotile exposure and not interference with cell attachment; the smaller reduction of colony formation displayed by the amphibole-exposed cells in these experiments suggests that the effect on cytotoxicity might be due in part to limitation of growth. Alternatively, recovery from amosite and crocidolite exposure may have been more rapid than recovery from chrysotile exposure.

As a further investigation into the nature of the asbestos-produced inhibition of colony formation, ARL-6 and I-407 cells were exposed to asbestos for 6 days at three concentrations of each fiber type (Table 3). Cells surviving exposure to the highest concentration were subsequently reseeded and reexposed to asbestos for another 6-day interval. The cytotoxicity as measured by the inhibition of colony formation following the second exposure to asbestos was usually greater in comparison to the cytotoxicity following the first exposure (Table 4). These results suggest that the cell population surviving asbestos exposure does not possess an inherent resistance to asbestos toxicity. Thus, colony formation at toxic doses of asbestos is not the result of the selection of a resistant subpopulation.

Cytotoxicity of Leached UICC Asbestos

Samples of asbestos were leached in sterile deionized water. The cytotoxic effects of the water-leached chrysotile, amosite, and crocidolite were not signifi-

TABLE 3
ASBESTOS TOXICITY IN RESEEDED CULTURES OF I-407 AND ARL-6 CELLS[a]

		Colony-forming efficiency (%)[b]			
		I-407 Cells		ARL-6 Cells	
	Concentration (%)	Control	Exposed	Control	Exposed
Amosite	10^{-2}	74 ± 3	48 ± 5[d]	57 ± 5	46 ± 5[d]
Crocidolite	10^{-2}	65 ± 7	38 ± 4[d]	47 ± 4	44 ± 3
Chrysotile	10^{-3}, 5×10^{-3}[c]	45 ± 3	19 ± 3[d]	64 ± 5	10 ± 2[d]

[a] Values were obtained by exposing I-407 and ARL-6 cells to asbestos for a 6-day interval; exposed and control cells were washed twice, and colonies were formed. When these colonies contained at least 50 cells/colony, they were trypsinized and reseeded. Colonies were permitted to develop for 1 to 2 weeks before counting.

[b] Each value represents the average CFE of ARL-6 or I-407 cells for six flasks; the standard deviations are included.

[c] For these experiments, I-407 cells were exposed to 10^{-3}% chrysotile, and ARL-6 cells were exposed to 5×10^{-3}% chrysotile.

[d] Significantly less than value for control with $P < 0.05$.

cantly different from unleached samples in ARL-6 cells (Table 5) or I-407 cells (Table 6). In a further attempt to diminish the cytotoxicity of asbestos, samples were leached in 5 N HCl for three consecutive 24-hr or one 96-hr interval. Chrysotile leached with 5 N HCl was significantly less toxic than either unleached

TABLE 4
ASBESTOS TOXICITY IN CULTURES OF ARL-6 AND I-407 CELLS FOLLOWING ONE AND TWO 6-DAY INTERVALS OF EXPOSURE TO ASBESTOS[a]

	Toxicity (%)[b]	
Asbestos concentration (%, w/v)	Following first exposure	Following second exposure
Chrysotile		
5×10^{-4}	49 ± 4	67 ± 8[c]
10^{-3}	77 ± 2	84 ± 6[c]
5×10^{-3}	95 ± 2	93 ± 7
Amosite		
2.5×10^{-2}	35 ± 8	54 ± 5[c]
5×10^{-3}	61 ± 5	83 ± 2[c]
10^{-2}	83 ± 1	93 ± 3[c]
Crocidolite		
10^{-3}	14 ± 6	41 ± 8[c]
5×10^{-3}	59 ± 5	95 ± 2[c]
10^{-2}	81 ± 2	99 ± 1[c]

[a] Inhibition of CFE of ARL-6 or I-407 cells following one and two 6-day intervals of exposure to asbestos. Following the first interval of exposure, cells surviving the highest concentration of asbestos were reseeded for the second interval of exposure.

[b] The values for chrysotile are from experiments using ARL-6 cells and the values for amosite and crocidolite are from experiments using I-407 cells; however, both cell lines were used to test all fiber types, and the values shown are representative of the results obtained from experiments using both cell lines. Each value represents the average toxicity for four to eight flasks; the standard deviations are included.

[c] Significantly greater than value for toxicity following first exposure.

TABLE 5
ASBESTOS TOXICITY IN CULTURES OF ARL-6 CELLS FOLLOWING LEACHING IN STERILE DEIONIZED WATER OR 5 N HCL

Asbestos	Experiment	Concentration (% w/v)	Toxicity[a]		
			Unleached	H_2O leached	5 N HCl leached
Amosite	1	2.5×10^{-2}	79 ± 8	55 ± 6[b]	54 ± 6
	2	2.5×10^{-2}	76 ± 8	78 ± 2	98 ± 1[c,d]
	3	2.5×10^{-1}	25 ± 5	25 ± 7	42 ± 6[c,e]
Crocidolite	1	2.5×10^{-2}	34 ± 7	42 ± 7	45 ± 12
	2	2.5×10^{-2}	61 ± 5	65 ± 3	95 ± 4[c,d]
	3	2.5×10^{-2}	12 ± 10	19 ± 6	36 ± 4[c,e]
Chrysotile	1	2.5×10^{-3}	93 ± 3	96 ± 3	29 ± 10[c]
	2	2.5×10^{-3}	92 ± 3	99 ± 1[b]	76 ± 12[c,d]
	3	3.3×10^{-3}	51 ± 8	51 ± 6	9 ± 7[c,e]

[a] Inhibition of CFE of ARL-6 cells following 3 days exposure to asbestos; each value represents the average percentage inhibition of CFE for seven flasks compared to seven control flasks not exposed to asbestos; the standard deviations are included. In experiment 1, samples were leached in water for three consecutive 24-hr intervals or in 5 N HCl for one 96-hr interval. In experiment 2, both water leaching and 5 N HCl leaching were performed for three consecutive 24-hr intervals. In experiment 3, both water leaching and 5 N HCl leaching were performed for one 96-hr interval.

[b] Significantly different from value for unleached with $P < 0.05$.

[c] Significantly different from value for H_2O leached with $P < 0.05$.

[d] Before addition to the culture medium, these asbestos samples were washed in 200 vol sterile deionized water and recentrifuged for 15 min at 10,000g.

[e] 5 N HCl was neutralized with 1 N NaOH before addition of asbestos to the culture medium.

or water-leached chrysotile; however, 5 N HCl-leached amphiboles were slightly more toxic, particularly to the ARL-6 cells, than water-leached amphiboles.

Despite the fact that water leaching did not produce a distinct change in the cytotoxicity of the three types of asbestos, atomic absorption spectrophotometry indicated that Mg^{2+} and Ca^{2+} were released from the asbestos fibers into the supernatant (Table 7). Thus, leaching in water did alter the chemistry of the asbestos fibers. During a 24-hr interval, a significantly greater titer of Mg^{2+} was released from chrysotile than from the amphiboles, and the order of Ca^{2+}-release was crocidolite > amosite > chrysotile. Leaching in 5 N HCl for a 24-hr interval removed more Mg^{2+} from all three asbestos types, particularly chrysotile. Ca^{2+} removal was also increased in 5 N HCl, especially from crocidolite. A longer interval (96 hr) of leaching in 5 N HCl further increased the removal of Mg^{2+} but not Ca^{2+} from all three asbestos types. To verify that the values obtained from spectrophotometric analysis were not due to asbestos fibers remaining in the centrifuged leaching fluid, samples of the fluid were filtered through sterile Nalgene filter units of pore size 0.2 or 0.45 μm. In all cases, the Mg^{2+} and Ca^{2+} values after filtration displayed the same trends, indicating that these values were not affected by contamination of the samples with particulate asbestos. Increased values following filtration of the 5 N HCl leaching fluid may have been due to extraction of atomic absorptive moieties from the filter membrane.

TABLE 6
ASBESTOS TOXICITY IN CULTURES OF I-407 CELLS FOLLOWING LEACHING IN STERILE DEIONIZED WATER OR 5 N HCL

Asbestos	Experiment	Concentration (%, w/v)	Toxicity[a]		
			Unleached	H_2O leached	5 N HCl leached
Amosite	1	10^{-3}	35 ± 10	35 ± 15	—
	1	2.5×10^{-3}	54 ± 10	56 ± 5	—
	2	2.5×10^{-3}	58 ± 9	47 ± 9[b]	23 ± 11[c,d]
	3	5×10^{-3}	97 ± 3	96 ± 3	99 ± 1[c]
	4	3.3×10^{-3}	80 ± 4	74 ± 5[b]	76 ± 4[d,e]
Crocidolite	1	10^{-3}	18 ± 10	15 ± 15	—
	1	2.5×10^{-3}	45 ± 13	39 ± 6	—
	2	2.5×10^{-3}	23 ± 7	27 ± 6	36 ± 6[c,d]
	3	5.0×10^{-3}	97 ± 3	88 ± 13	92 ± 3
	4	3.3×10^{-3}	62 ± 4	60 ± 10	66 ± 7[d,e]
Chrysotile	1	10^{-4}	24 ± 19	37 ± 8	—
	1	2.5×10^{-4}	31 ± 17	39 ± 12	—
	2	2.5×10^{-4}	25 ± 9	35 ± 7[b]	5 ± 9[c,d]
	3	5×10^{-4}	91 ± 5	94 ± 1	48 ± 8[c,e]
	4	5×10^{-4}	87 ± 8	71 ± 6[b]	31 ± 10[c,e]

[a] Inhibition of CFE I-407 cells following 3 days exposure to asbestos; each value represents the average percentage inhibition of CFE for seven flasks compared to seven control flasks not exposed to asbestos; the standard deviations are included. All samples were leached for three consecutive 24-hr intervals except in experiment 2 where one 96-hr interval of 5 N HCl leaching was used and experiment 4 where one 96-hr interval of water and 5 N HCl leaching were used.

[b] Significantly different from value for unleached with $P < 0.05$.

[c] Significantly different from value for H_2O leached with $P < 0.05$.

[d] Before addition to the culture medium, these asbestos samples were washed in 200 vol sterile deionized water and recentrifuged for 15 min at 10,000g.

[e] 5 N HCl was neutralized with 1 N NaOH before addition of asbestos to the culture medium.

DISCUSSION

Three forms of UICC asbestos, amosite, crocidolite, and chrysotile, were analyzed for their cytotoxicity using a quantitative assay for inhibition of colony formation. When assayed on I-407, ARL-6, and MCE-1 epithelial cells, all three forms of asbestos produced marked cytotoxicity. Chrysotile was approximately tenfold more toxic than the amphiboles. All three asbestos types, particularly the amphiboles, were more toxic to I-407 than ARL-6 cells. The MCE-1 cells were less sensitive to all fiber types than the I-407 cells; although these cells exhibited a level of susceptibility to chrysotile and amosite similar to the ARL-6 cells, they were more sensitive to crocidolite. The reproducibility of the results from the cytotoxicity assay demonstrates the usefulness of assaying for inhibition of colony formation as a method for the quantitation of asbestos toxicity.

Our results correspond to the data of other investigators indicating chrysotile to be more inhibitory than the amphiboles to macrophage (Bey and Harington, 1971; Wade *et al.*, 1976) and epithelial cell (Neugut *et al.*, 1978) growth. Chamberlain and Brown (1978), however, using as a criterion the cloning efficiency of V79-4

TABLE 7

RELEASE OF Mg^{2+} AND Ca^{2+} INTO STERILE DEIONIZED WATER OR 5 N HYDROCHLORIC ACID BY ASBESTOS[a]

Asbestos	Experiment	Leaching fluid	Leaching time (hr)	Mg^{2+}		Ca^{2+}	
				Unfiltered	Filtered[b]	Unfiltered	Filtered[b]
Amosite	1	Sterile deionized water	24	—	35	62	107
	2	Sterile deionized water	24	22	92	95	91
	3	5 N hydrochloric acid	24	394	—	119	—
	4	5 N hydrochloric acid	96	474	546	91	206
Crocidolite	1	Sterile deionized water	24	10	27	103	107
	2	Sterile deionized water	24	10	60	144	86
	3	5 N hydrochloric acid	24	222	—	1,170	—
	4	5 N hydrochloric acid	96	282	344	807	2,450

Chrysotile	1	Sterile deionized water	24	549	499	37	206
	2	Sterile deionized water	24	499	1,030	37	235
	3	5 N hydrochloric acid	24	20,400	—	50	—
	4	5 N hydrochloric acid	96	34,700	33,900	66	181
None	1	Sterile deionized water	24	0	3	0	17
	2	Sterile deionized water	24	0	0	0	0
	3	5 N hydrochloric acid	24	0	—	0	—
	4	5 N hydrochloric acid	96	0	—	0	—

[a] Values for the concentrations of Mg^{2+} and Ca^{2+} were obtained in parts per million (ppm) by atomic absorption spectrophotometric analysis of leaching-fluid samples following 24 hr (experiments 1, 2, and 3) or 96 hr (experiment 4) of leaching at 25°C at 0.5% (w/v). Each sample was monitored using reference standards of known Mg^{2+} or Ca^{2+} ppm and then converted to micromoles by division with 0.0401 (for Mg^{2+} values) or 0.0243 (for Ca^{2+} values).

[b] Leaching fluid filtered through a Nalgene 0.2 (experiments 1 and 3) or 0.45 μm (experiments 2 and 4) filter unit before atomic absorption spectrophotometric analysis.

Chinese hamster lung cells that were seeded together with asbestos, found the order of asbestos cytotoxicity to be amosite > crocidolite > chrysotile. Using cell growth in mass culture, they found, as others have, that chrysotile was more inhibitory than crocidolite to A-549 human alveolar lung cells; inhibition of cell growth in the presence of amosite was not determined by this group. A possible explanation for the reversed order of toxicity obtained by Chamberlain and Brown in their cloning efficiency assay may derive from the fact that they studied simultaneous addition of cells and asbestos fibers to the culture dishes (in some cases a 2.5-hr delay between seeding and addition of the fibers was used). We found that the presence of fibers on the surface of a flask was inhibitory to cell attachment; furthermore, the order of adherence of asbestos fibers to the culture surface was amosite > crocidolite > chrysotile. Thus, the values obtained by Chamberlain and Brown for cloning efficiency appear to be a composite of values for attachment efficiency and colony-forming efficiency in the presence of asbestos fibers and may not represent asbestos cytotoxicity alone. In our assay system, the asbestos was added 24 hr following seeding in order to minimize the growth-limiting effect of adherent fibers. Although some growth limitation due to nontoxic effects of the fibers may still have occurred, delayed addition of the fibers decreased the apparent toxicity of the adherent amosite fibers. The cytotoxicities of amphiboles (crocidolite, amosite, and anthophyllite) containing decreasing percentages of long fibers were also compared by these workers, using the same methodology (Brown *et al.*, 1978). Decreased colony number, due to inhibition of cell attachment and cytotoxicity, was largely dependent upon the presence of fibers of at least 6.5 μm in length.

The comparative toxicities of three forms of asbestos on three different cell lines were examined in the present study. Except for the study by Neugut *et al.* (1978) on inhibition of mass growth, ours is the first description of a comparative analysis of asbestos cytotoxicity performed in more than one cell line. As can be seen from the results of Neugut's study and ours, the toxicity of each form of asbestos varies from one line to another, although for each cell type the order of toxicity of the different forms of asbestos was constant. The ARL-6 line was only slightly less sensitive to asbestos than the I-407 and MCE-1 lines. Since the ARL-6 line is known to be active in the metabolism of organic carcinogens, these data do not suggest that asbestos cytotoxicity results from the metabolism of activation dependent components, nor does the cytotoxic principle appear to be highly metabolically degradable.

In order to determine whether asbestos cytotoxicity is due entirely to the asbestos fibers themselves, or to extractable moieties bound to these fibers, we leached the asbestos in sterile deionized water and 5 N HCl. Leaching of amosite, crocidolite, and chrysotile for 3 days in sterile deionized water did not appreciably affect the toxicity of the three asbestos forms, although analysis of the postleaching fluid revealed that extraction of Mg^{2+} particularly from chrysotile, and Ca^{2+}, particularly from crocidolite, had occurred. Preferential Mg^{2+} leakage from chrysotile and preferential Ca^{2+} leakage from the amphiboles, particularly crocidolite, during aqueous leaching was also noticed by Pelfrene (1977). Leaching in 5 N HCl greatly decreased the toxicity of chrysotile and particularly in

cultures of ARL-6 cells, slightly increased the toxicity of amosite and crocidolite. A sizeable loss of Mg^{2+} and Ca^{2+} from all three asbestos fiber types with preferential Mg^{2+} leakage from chrysotile and preferential Ca^{2+} leakage from crocidolite was produced by leaching in 5 N HCl. These results suggest relationships between the efficacy of leaching chrysotile and its loss of cytotoxicity and between the efficacy of leaching the amphiboles and their increased cytotoxicity.

The changes produced in the toxicity of asbestos by leaching could be attributed to Mg^{2+} and Ca^{2+} depletion. Mg^{2+} leaching of chrysotile would increase the structural similarity of this form of asbestos to silica; furthermore, Mg^{2+}-leached chrysotile and silica have similar biological properties (Morgan *et al.*, 1977). Light and Wei (1977a) found that leaching progressively decreased the surface charge and hemolytic activity of chrysotile but increased these properties for the amphiboles. The convergence of activities seen in their results as well as in the present studies may explain why the serpentine and amphibole classes of asbestos fibers, although different in their short-term effects (Schnitzer and Pundsack, 1970; Desai *et al.*, 1975; Harington *et al.*, 1975), are not clearly different in their long-term effects (i.e., delayed cytotoxicity, fibrogenicity, and tumorigenicity) (Stanton and Wrench, 1972; Wagner *et al.*, 1973, 1974; Wade *et al.*, 1976; Light and Wei, 1977a).

Other ionic or molecular species besides Mg^{2+} and Ca^{2+} were undoubtedly released during leaching. Langer (1974) reported evidence of iron loss from asbestos fibers leached geologically; *in vivo*, magnesium (Jaurand *et al.*, 1977), as well as trace elements such as cobalt, chromium, and nickel (Morgan *et al.*, 1971; Zaidi *et al.*, 1976), was removed from asbestos. Therefore, an analysis of the water- and acid-postleaching fluids for their total contents following chrysotile leaching would be necessary to definitively identify an agent contributing to chrysotile toxicity. Any substance present in the acid-extracted fraction in a significantly greater concentration than that in the water-extracted fraction might be a factor involved in chrysotile activity. Conversely, any factor present in the acid- but not the water-extracted fraction from the amphiboles may be responsible for blocking sites involved in the toxicity of this group of fibers.

Chrysotile and crocidolite have been reported to induce acute, but not heritable, chromosome damage in cultured Syrian hamster embryo cells (Lauappa *et al.*, 1975), Chinese hamster ovary cells (Sincock and Seabright, 1975), murine 3T3 cells (Sincock, 1976), and Chinese hamster lung cells (Huang *et al.*, 1978). Although no mutagenic activity was found to be associated with amosite, crocidolite, or chrysotile in mutation tests using *E. coli* or *S. typhimurium* (Chamberlain and Tarmy, 1977), crocidolite was reported to have weak mutagenic activity at the hypoxanthine–guanine phosphoribosyl transferase locus in cultures of Chinese hamster lung cells (Huang *et al.*, 1978). We also found no substantial mutagenicity of asbestos to the ARL-6 cell line (Reiss *et al.*, 1979). The lack of definitive mutagenic activity that can be attributed to asbestos suggests that asbestos does not operate primarily as a genotoxic carcinogen that produces chemical alterations in DNA (Williams, 1979) but may be classified as an epigenetic carcinogen of the solid state type (Weisburger and Williams, 1979).

The production of tumors by implanted glass and asbestos fibers has been

shown to be a function of fiber size (Stanton and Wrench, 1972; Pott *et al.*, 1976; Stanton *et al.*, 1977; Brown *et al.*, 1978); however, the fact that removal of surface ligands from the fibers by leaching in water or acid influences the cytotoxic (Light and Wei, 1977a, b; Pelfrene, 1977) and tumorigenic (Morgan *et al.*, 1977) potential of the asbestos fibers suggests that asbestos cytotoxicity and tumorigenicity may involve both structural (fiber size) and physicochemical (reactivity at specific sites on the fibers) mechanisms.

Mg^{2+}, believed to be an important ion in hemolysis (Desai *et al.*, 1975; Harington *et al.*, 1975; Harington, 1976), is also implicated in mechanisms of action proposed to explain asbestos carcinogenicity. These mechanisms emphasize the reaction of surface ligands, such as Mg^{2+}, of phagocytosed asbestos fibers with lysosomal membrane sialoglycoproteins (Davies *et al.*, 1974; Harington *et al.*, 1975; Harington, 1976; Tetley *et al.*, 1976, 1977). Such mechanisms, however, have not been confirmed, nor do they include the ultimate production of tumors. It is, therefore, necessary for specific reactive groups on asbestos fibers to be identified and the effects of these groups on cells to be elucidated. Related phenomena, such as the biological coating of asbestos with protective proteins that reduce asbestos toxicity (Desai *et al.*, 1975), also require explanation.

The cytotoxicity assay used in this investigation provides a rapid and reliable method for quantitating the inhibitory effect of asbestos on colony formation in mammalian cells. This assay measures physical alterations produced by asbestos in cells, and therefore, may also be a suitable assay for the evaluation of asbestos as a solid state carcinogen.

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